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Study on the response mechanism of MicroRNA *novel-13* and *novel-44* to *Vibrio parahaemolyticus* infection in *Pinctada fucata martensii*

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Abstract

Pinctada fucata martensii (*P. f. martensii*) is one of the main pearl oysters cultured in artificial seawater in China. However, it is highly susceptible to pathogen infection under intensive cultivation near the coast. MicroRNAs (miRNAs), as an innovative and potent regulator of immune function, play a pivotal role in the immune response of pearl oysters to external stimuli and are a potent marker for the response of *P. f. martensii* to infection. This study identified two novel miRNAs, *novel-13* and *novel-44*, from the whole transcriptome of the *P. f. martensii* hemocyte before and after infection with *Vibrio parahaemolyticus*. The dual luciferase results showed that *novel-13* negatively regulated *LAAO* and *novel-44* negatively regulated *ILK*. The activity of antioxidant-related enzymes increased significantly in the synthetic miRNA (*novel-13* and *novel-44*) inhibitors and decreased significantly in the synthetic miRNA mimics. In the challenge experiment, injection with miRNA inhibitor increased the relative survival percentage by 10% compared with the control group. In conclusion, the overexpression of *novel-13* and *novel-44* can decrease the activity of immune and antioxidant-related enzymes, possibly affecting immune regulation in *P. f. martensii* by negatively regulating the *LAAO* and *ILK* target genes.

Keywords *Pinctada fucata martensii*, miRNAs, Target gene, *Vibrio parahaemolyticus*

Introduction

MicroRNAs (miRNAs) are highly conserved single-stranded non-coding RNAs, usually 18–25 nucleotides (nt) long. These miRNAs are widely distributed in plants, animals and viruses, indicating their critical role in the post-transcriptional regulation of genes [1]. MicroRNAs regulate gene expression by linking to complementary sequences of the 3' or 5' untranslated regions (3' UTR or 5' UTR) of the target mRNAs, blocking translation mechanisms and driving mRNA cleavage [2]. Some studies have shown that miRNAs can target the coding sequence (CDS) of mRNA, depending on the pairing of the 3' end of the miRNAs with the target [3]. The first miRNA, *Lin-4*, discovered in *Caenorhabditis elegans* (*C. elegans*), can adjust nematode growth and development [4]. Subsequently, *lin-4* RNA pairs with the mRNA sequence of *lin-14*, another gene in the developmental system of *C. elegans*, to control the production of the *lin-14* protein. A second miRNA (*let-7*) is conserved in many organisms,

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including humans, suggesting that its regulatory mechanism has a broader role in biology [5, 6].

Subsequently, thousands of miRNAs have been discovered in many organisms, based on the prediction that each miRNA can regulate hundreds of target genes, and the entire miRNA pathway is a key mechanism for controlling gene expression [7]. The unique gene silencing mechanism makes miRNAs crucial in organ development, signal transduction, cell proliferation, apoptosis, tumor occurrence, hematopoiesis, innate immunity, and adaptive immunity [8, 9]. In vitro experiments with *miR-223* have demonstrated enhanced granulocyte generation. Mice lacking *miR-223* exhibit heightened inflammatory lung lesions and tissue damage upon endotoxin stimulation [10, 11]. In the case of *Larimichthys crocea*, *miR-7132c* can downregulate *TLR5S*, influencing immune response through the TLR5S-mediated signaling pathway [12]. In *Apostichopus japonicus*, *miR-2008* regulates skin ulcer syndrome by targeting *TLR3* [13], and *miRNA-133* augments luminal cell phagocytosis of the challenge bacteria by targeting the *TLR* components of *IRAK-1* [14].

Previous studies have demonstrated that miRNAs are involved in the immune defense of *Pinctada fucata martensii* (*P. f. martensii*). For instance, Zhang obtained 186 miRNAs from the hemocyte miRNA transcriptome after the nuclear implantation of *P. f. martensii*. Furthermore, miR-1985 may regulate *GATA-3* and *IFI44L*, which are involved in the inflammatory response of *P. f. martensiii*, after implanted nucleus surgery. *MiR-184-3p* can alleviate the rejection reaction caused by nuclear transplantation by regulating *TLR3* and *FN*. Moreover, *novel-63* may enhance the immune response of *P. f. martensii* by regulating *GDP-FucTP*, *CysLTR2* and *RLR-like*, to enhance interferon and inflammatory cytokine production after nucleus implantation [15].

Pearls cultivated by *P. f. martensii*, also known as "southern pearls", account for over 95% of the Chinese seawater pearls [16]. In recent years, the high density of aquaculture, deterioration of sea quality, and frequent disease outbreaks have killed numerous *P. f. martensii*, greatly threatening the sustainable aquacultural development and yield of marine pearls [17]. The immune mechanism of *P. f. martensii* is non-specific, involving humoral immunity and hemocyte immunity [18]. The hemocyte is the main organ of immune defense, involving phagocytosis and cyst function, and producing and releasing various immune factors to participate in humoral immunity. They also participate in humoral immunity, thus combining cellular and humoral immunity. Smith [19] showed that shellfish hemocytes participate in a series of immune reactions, including phagocytosis, encapsulation, nodule formation, inflammation, wound repair, and phagocytosis of foreign bodies and soluble proteins [20].

Vibrios are Gram-negative rod-shaped bacteria that are natural constituents of estuarine and marine environments and can cause disease in a variety of marine organisms [21]. More specifically, they cause different degrees of damage to shellfish at all growth stages [22]. Among Vibrios, *Vibrio parahaemolyticus* (*VP*) is of particular concern because it causes large-scale disease outbreaks in marine organisms through direct contact and large foodborne disease outbreaks in humans through consumption [23, 24]. A previously generated miRNA transcriptome library of the pearl oyster after *VP* infection showed that the infection up-regulated 15 and down-regulated 51 miRNAs compared with the control group [25]. Two novel miRNAs (*novel-13* and *novel-44*) selected from the differentially expressed genes (DEGs) were significantly down-regulated after *VP* infection. To further explore the role of these two miRNAs after *VP* infection in the pearl oyster, the target relationship was verified by dual luciferase assay. The activities of the immune and antioxidant-related enzymes of *P. f. martensii* were detected by injecting inhibitors and mimics of the two miRNAs before and after *VP* infection. The purpose of the study was to further explore the role of *P. f. martensii* miRNAs (*novel-13* and *novel-44*) in *VP* infection.

Materials and methods

Prediction of the binding sites of *novel-13* with *LAAO* and *novel-44* with *ILK*

The differentially expressed miRNAs (*novel-13* and *novel-44*) and their target binding sites were detected using the miRanda (http://www.bioinformatics.com.cn/) online tool.

Design and synthesis of the target genes wild-type (WT) and mutant (MUT) binding site sequences

The binding site sequences of the relevant target genes were obtained by whole gene synthesis and target prediction using ~ 200 bp target gene binding site sequences as synthetic sequences. Primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd., who added NheI and BglII enzyme loci to protect the base. The steps were as follows.

The synthesized primers were dissolved in enzyme-free water, and the same volume of both primers was mixed in a new centrifuge tube to obtain an oligo mix. This was followed by a first-round PCR reaction with the following reaction system: Pfu DNA polymerase, 0.2 μg; 10×Pfu Buffer (+ Mg^{2+}), 3 μL; Oligo mix, 2 μL; dNTP, 0.6 μL; DMSO, 1.2 μL; and DEPC water, 23 μL. The PCR procedure was as follows: pre-denaturation at 95 ℃ for 180 s; denaturation at 95 ℃ for 22 s, annealing at 58 ℃ for 22 s, and extension at 72 ℃ for 40 s, for 22 cycles. The extension was extended at 72 ℃ for 5 min. The second PCR reaction was as follows: Pfu DNA polymerase, 0.3 μg;

10×Pfu Buffer (+Mg^{2+}), 5 μL; first-round PCR products, 1 μL; primers, 1 μL; dNTP, 1 μL; DMSO, 2 μL; and DEPC water, 39 μL. The second PCR reaction followed the same thermal cycling protocol as the first round PCR. The PCR products were then purified and recovered with a DNA purification kit (Thermo Fisher Scientific), following the manufacturer's instructions.

Construction of the recombinant plasmid WT and MUT-associated binding sites of *LAAO* and *ILK*

The recombinant plasmid sequences of the binding sites (WT and MUT) of the target genes (*LAAO* and *ILK*) were designed using the Targetscan database and bioinformatics software to include the relevant binding sites and the NheI and BglII restriction sites. The binding sites of *LAAO* and *ILK* (WT/MUT) and the pmirGLO vector were subjected to NheI and BglII enzyme digestion, and the products were purified and recovered. The product was then ligated to the vector using T4 DNA ligase, and the plasmid was transformed into competent cells (DH5α). Recombinant plasmids were subsequently obtained using a plasmid extraction kit (EasyPure Plasmid Miniprep, Beijing TransGen Biotech Co., Ltd.).

Cell culture, cell transfection, and dual luciferase assay

The HEK 293T cells were removed from the − 80℃ refrigerator and placed in a 37 ℃ thermostatic water bath. When the cells were nearly completely defrosted, they were removed and covered in a DEME (high glucose) medium containing 10% FBS. Then, the cells were placed in a cell thermostatic incubator and passaged every 2–3 days to retain the adherent cells. The cell passage conditions were based on cell growth, thus, they were passaged when the cells accounted for 85% of the bottle wall. The passage was repeated until the cell vitality increased and then the passaged cells were inoculated into a 96-well plate. When the cell growth to hole reached 80 – 90%, the culture medium was absorbed and the cells were slowly washed with filtered PBS. Next, a 3 pmol microRNA mimic was added to each pore, followed by 0.2 μg of the corresponding dual fluorescence reporter carrier following the Lipofectamine 3000 reagent specifications and the recommendation for using Gima RNAi. Each miRNA was administered in three groups: double fluorescence reporter vector WT + mimic, double fluorescence reporter vector WT + NC mimic, and double fluorescence reporter vector MUT + mimic. Next, 100 μL of the serum-free culture medium was added to each well of the 96-well plate, and the cells hunger-treated for 2 h to join the complex to the cells. The plate was then left for 4 h to continue developing. The old culture was discarded, and the cells were mixed with 60 μL fresh culture and left for 42 h to continue developing. Subsequently, the RLU value was detected by the Dual-LumiTM II dual-luciferase reporter gene detection kit (Shanghai Biyuntian Biological Co., Ltd.), and the relative luciferase activity was recorded and analyzed.

Table 1 The primers required for the experiment

Primers	Sequences (5′-3′)	purposes
U6-R	ATTTGCGTGTCATCCTTGC	Quantitative real-time PCR
U6-F	ATTGGAACGATACAGAGAAGATT	Quantitative real-time PCR
novel-13	UCACAGCCAGUUUUGAUGAGCC	Quantitative real-time PCR
novel-44	GAUGUUGGCCGUGUCUGUGACU	Quantitative real-time PCR
NC	UUCUCCGAACGUGUCACGUTT	Overexpression
novel-13 mimics	UCACAGCCAGUUUUGAUGAGCC	Overexpression
novel-13 inhibitor	GGCUCAUCAAAACUGGCUGUGA	Overexpression
novel-44 mimics	GAUGUUGGCCGUGUCUGUGACU	Overexpression
novel-44 inhibitor	AGUCACAGACACGGCCAACAUC	Overexpression

Sample preparation

Experimental *P. f. martensii* (about 2 years old) was obtained from Xuwen County, Zhanjiang City, Guangdong Province, China. Before the experiment, the *P. f. martensii* were temporarily raised in a seawater tank containing rich oxygen for three days to adapt to the water temperature (20–25 ℃). The *chlorella* was fed once every three days, and the water was changed once every two days.

Functional validation of *novel-13* and *novel-44* before and after *VP* infection

Before *VP* infection, the miRNA mimics, inhibitor mimics, and NC (Negative Control) mimic (Table 1) used for in vivo expression were ordered from Shanghai Jimma Company. The experimental groups, each containing 13 shellfish, included the *novel-13* mimic, *novel-13* inhibitor, *novel-44* mimic, *novel-44* inhibitor, NC, and PBS control. Next, 100 μL of each reagent was injected per shellfish at a concentration of 10 μg/μL according to the RNA oligo synthesis report. Gill tissues were harvested 12 h after injection, snap-frozen in liquid nitrogen, and stored at -80℃. The enzyme activity in the gill tissues was detected using the Enzyme Activity Test Kit of Nanjing Jiancheng Institute of Biological Engineering, following the instruction manual. The activities of six enzymes, namely acid phosphatase (ACP), lysozyme (LZM), total antioxidant enzyme (T-AOC), total superoxide dismutase (SOD), catalase (CAT), and alkaline phosphatase (ALP/AKP) were detected in shellfish after injection with *novel-13* mimic, *novel-13* inhibitor, *novel-44* mimic, *novel-44* inhibitor, NC and PBS.

Twenty-four hours after the injection of the mimics and inhibitors, each shellfish was injected with *VP* (80μL, 2.3×10^{12} CFU/mL) following the grouping and injection

dose before *VP* infection. Gill tissues were harvested 12 h after injection, snap-frozen in liquid nitrogen, and stored at -80 ℃. Next, the enzyme activities in the gill tissues were measured as described above.

Real-time fluorescence quantification of *novel-13* and *novel-44*

The gill tissues collected from the above experiments were used for RNA extraction using Trizol reagent (Life Technologies). The RNA was reverse transcribed into cDNA using the miRcute enhanced miRNAs cDNA first-strand synthesis kit (Tiangen Biotech (Beijing) Co., Ltd.), following the manufacturer's instructions. Fluorescence quantification was performed using the miRcute Plus miRNAs fluorescence quantitative detection kit (Tiangen Biotech (Beijing) Co., Ltd.), following the manufacturer's instructions, with *U6* as the reference gene.

Survival rates test

Here, the number of shellfish in each group was increased to 30, following the experimental grouping, injection dose, and concentration stated in Sect. 2.2. The samples were observed for 14 d to detect the morbidity and mortality of infected shellfish. Pathological anatomy and pathogen detection were performed on dying and dead shellfish to determine whether they died from the *VP* infection. The survival rates of each group were calculated after the experiment as follows: SR (%) = number of surviving shellfish/initial number of shellfish × 100.

Data statistics and analysis

All the experimental data were normalized and all analyses were performed in SPSS2.0. Fluorescence quantification was performed using the $2^{-\Delta\Delta ct}$ method to calculate relative expression. One-way analysis of variance was used to test significance and the LSD and Duncan tests were used for post hoc multiple analyses. There were three replicates per group, and the results were presented as mean ± standard deviation.

Results

Target gene prediction results

The results showed that *novel-13* and *novel-44* had potential interaction sites with *LAAO* and *ILK*, respectively, which were potential target genes. The results of the website projections are shown in the Supplementary material.

Target gene validation

The results of the dual luciferase assay (Fig. 1) showed that the luciferase activity of the wild-type co-transfection group (*LAAO*-pmirGLO-WT + *novel-13* mimic and *ILK*-pmirGLO-WT + *novel-44* mimic) was significantly lower than that of the control group (*LAAO*-pmirGLO-WT + NC and *ILK*-pmirGLO-WT + NC) and the mutant co-transfection group (*LAAO*-pmirGLO-MUT + *novel-13* mimic and *ILK*-pmirGLO-MUT + *novel-44*). Therefore, *novel-13* and *novel-44* could bind to the *LAAO* and *ILK*-related sequences to down-regulate the expression of their target genes.

Functional validation of *novel-13* and *novel-44*

Novel-13 and novel-44 were overexpressed and repressed in vivo

Figure 2A shows the expression of *novel-13* (measured by qRT-PCR) in groups injected with the *novel-13* mimic, *novel-13* inhibitor, NC, and PBS. Figure 2B shows the expression of *novel-44* in the groups injected with *novel-44* mimic, *novel-44* inhibitor, NC, and PBS. The expression rate of *novel-13* and *novel-44* showed no significant difference between the NC and control groups. Compared with the NC control group, the expression rates of miRNAs in the *novel-13* mimic and *novel-44* mimic groups were upregulated by 2.1 times ($p < 0.05$) and 11.9 times ($p < 0.01$), respectively (Fig. 2A). Compared with

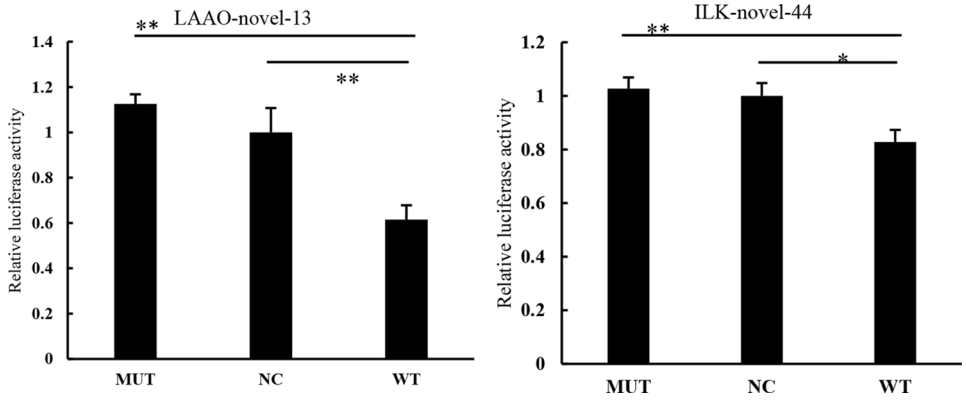

Fig. 1 *Novel-13* and *novel-44* Luciferase activity assay. *Note*: Data are presented as mean ± SD ($N = 3$). **A**: *novel-13* and *LAAO*, **B**: *novel-44* and *ILK*, * represents a significant difference with WT ($p < 0.05$), and ** represents a highly significant difference with WT ($p < 0.01$)

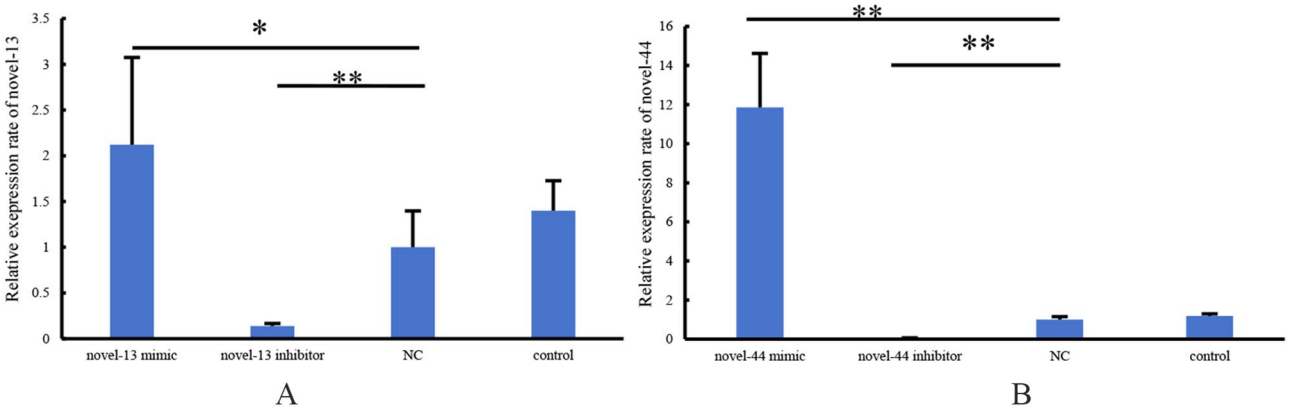

Fig. 2 Overexpression and inhibition of miRNAs in the gill tissues of *Pinctada fucata martensii*. *Note*: Data are presented as mean ± SD ($N = 3$). * Represents a significant difference compared to the NC control ($p < 0.05$), and ** represents a highly significant difference compared to the NC control ($p < 0.01$). Control: PBS control group; **A**: Overexpression and inhibition of novel-13 expression. **B**: Overexpression and inhibition of *novel-44*

the NC group, the expression rates of *novel-13* and *novel-44* in the *novel-13* inhibitor and *novel-44* inhibitor injection groups were downregulated 1.4 times ($p < 0.01$) and 0.1 times ($p < 0.01$) (Fig. 2B), respectively.

Immune and oxidation resistance-related enzyme activity

The activity of the six tested enzymes did not differ significantly between the NC and control groups (Fig. 3). Overexpressing the *novel-13* mimic significantly downregulated the activities of AKP, CAT, and T-AOC ($p < 0.05$). Injection of the *novel-13* inhibitor significantly upregulated the activities of ACP, AKP, CAT, SOD, T-AOC and LZM ($p < 0.05$). Compared to the NC group, overexpressing the *novel-44* mimic significantly downregulated the activities of AKP, CAT, SOD, and T-AOC ($p < 0.05$ or $p < 0.01$), while injection with the *novel-44* inhibitor significantly upregulated the activities of ACP, AKP, T-AOC, and LZM ($p < 0.05$).

Functional analysis of *novel-13* and *novel-44* in response to *VP*

Novel-13 and novel-44 were overexpressed and repressed in vivo

The expression rates of both *novel-13* and *novel-44* were not significantly different between the NC and control groups. Compared with the NC control group, the expression rates of *novel-13* and *novel-44* in the *novel-13* mimic and *novel-44* mimic groups were increased 2.7 times ($p < 0.05$) and 48.6 times ($p < 0.01$), respectively (Fig. 4A). Compared with the NC control group, the expression rates of *novel-13* and *novel-44* in the *novel-13* inhibitor and *novel-44* inhibitor injection groups were downregulated 0.3 times ($p < 0.01$) and 0.2 times ($p < 0.01$), respectively (Fig. 4B).

Immune-related enzyme activity

The activities of the six measured enzymes were not significantly different between the NC and control groups (Fig. 5). However, overexpressing the *novel-13* mimic significantly downregulated the activities of ACP, AKP, CAT, and T-AOC ($p < 0.05$ or $p < 0.01$) compared to NC. The activities of the six enzymes in the inhibitor group were significantly upregulated ($p < 0.05$ or $p < 0.01$). Meanwhile, overexpressing *novel-44* mimic significantly downregulated the activities of AKP, CAT, SOD, and T-AOC ($p < 0.05$ or $p < 0.01$) compared to NC, while the ACP, AKP, SOD, and LZM activities in the inhibitor group were significantly upregulated ($p < 0.05$ or $p < 0.01$).

Survival rates test

The oyster survival rates of each group within the designated 14 d are shown in Fig. 6. At 14 d, the survival rate of the injected inhibitor group was higher than that of the mimics and control groups, with the survival rates of the mimics injected with *novel-13* and *novel-44* being 23% and 27%, respectively. The survival rates of the inhibitor groups injected with *novel-13* and *novel-44* were 47% and 53%, respectively, and that of the NC-injected control group was 37%.

Discussion

MicroRNAs play an important role in the upstream regulation mechanism of most genes. They inhibit or degrade target genes by incomplete binding or complete pairing, and participate in post-transcriptional gene regulation, affecting the physiological and biochemical processes of organisms [1]. Previous work on immune regulation in the pearl oyster showed that miRNAs regulate immune response, where overexpressing *miR-29a* and *miR-146a* negatively regulated the expression of the neuroimmune system-related *Y2R* gene and the macrophage migration inhibitory factor (MIF). Both *miR-29a* and *miR-146a*

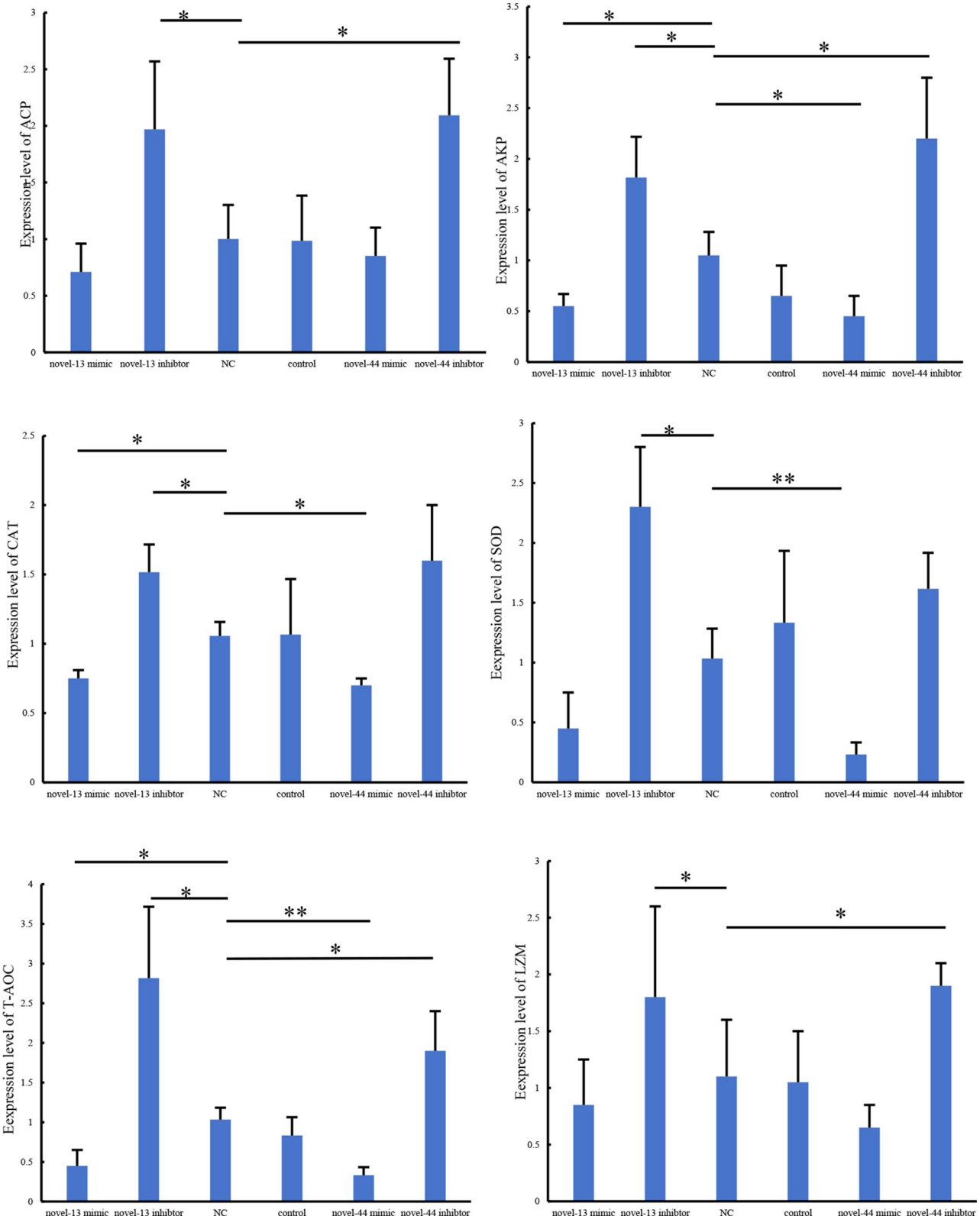

Fig. 3 Enzyme activity in the gill tissues in *Pinctada fucata martensii* after overexpression and inhibition of miRNA. *Note*: Data are presented as mean ± SD ($N = 3$). * Represents a significant difference with the NC control ($p < 0.05$), ** represents a highly significant difference with the NC control ($p < 0.01$), Control: PBS control group

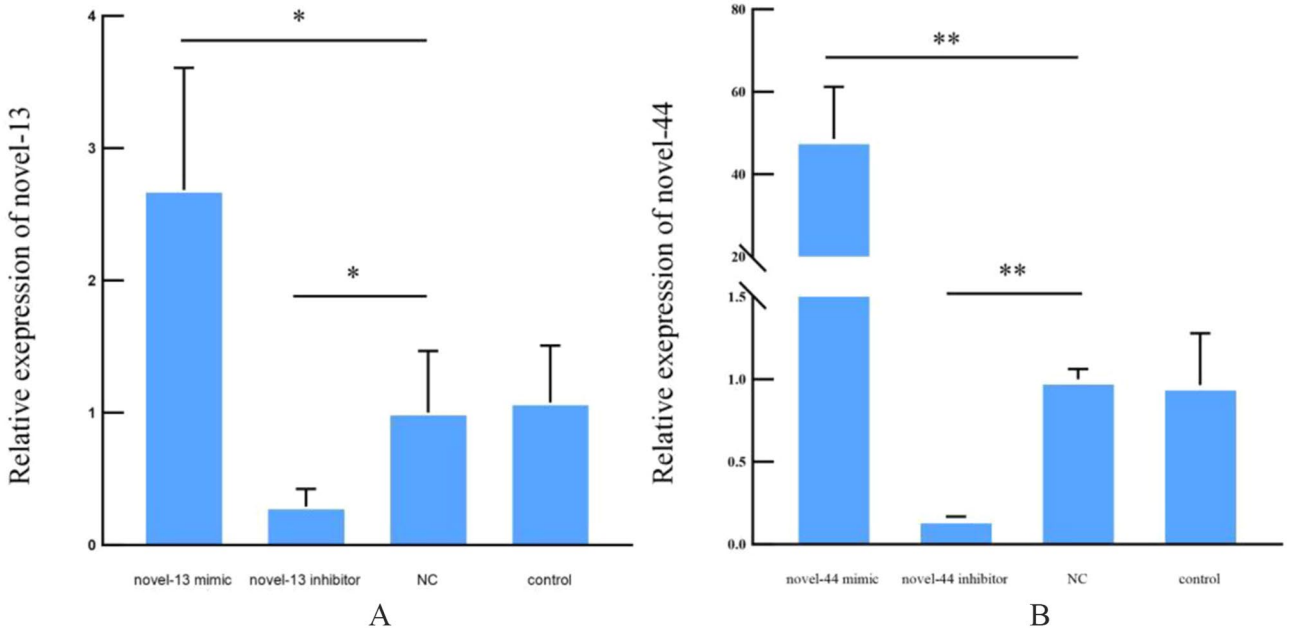

Fig. 4 Overexpression and inhibition of miRNAs during *Vibrio parahaemolyticus* stimulation in *Pinctada fucata martensii*. *Note*: Data are presented as mean ± SD (*N* = 3). * Represents significant difference with NC control group $p < 0.05$, ** represents highly significant difference with NC control group, $p < 0.01$. Control: PBS control group; **A**: Overexpression and inhibition of *novel-13*; **B**: Overexpression and inhibition of *novel-44*

affect the NF-κB signaling pathway, suggesting their involvement in immune regulation [26, 27].

Based on the combined analysis of the miRNAs transcriptome and the mRNA transcriptome of hemocytes after *VP* infection, it was found that *novel-13* and *novel-44* may target *LAAO* and *ILK*, respectively, and that there is a negative regulatory relationship [25]. Furthermore, the dual luciferase assay showed that *novel-13* and *novel-44* could recognize the *LAAO* and *ILK* binding sites. Co-transfection of *LAAO*-PmirGLO-WT and *ILK*-PmirGLO-WT with the *novel- 13* mimic and *novel-44* mimic, respectively, resulted in a significant drop in luciferase activity, with significant differences compared to the control group (mimic NC) and the mutant plasmid (MUT). These results indicate that *LAAO* and *ILK* are the target genes of *novel-13* and *novel-44*, respectively, and are negatively regulated by them. Also, *LAAO* inhibits bacteria by producing H_2O_2, while *ILK* regulates cancer cell migration and invasion mainly through the NF-κB pathway [28–30]. Therefore, *novel-13* and *novel-44* may be involved in the immune response of *P. f. martensii* by regulating their target genes. Similarly, in *Cynoglossus semilaevis*, *Cse-miR-33* can negatively regulate *CsTRAF6* to participate in the immune response against bacteria and viruses [31].

In marine shellfish, different miRNAs regulate osmotic pressure stress, temperature stress, heavy metal stress, oxidative stress, and stress from adaptive adjustment to all kinds of environments [32]. Not surprisingly, miRNAs are also involved in the immune response of *P. f. martensii* [26]. In this study, *novel-13* and *novel-44* were overexpressed and suppressed in healthy and *VP*-infected *P. f. martensii* to investigate their roles in its immune response. The miRNAs fluorescence quantification results indicated a successful in vitro injection, and that the resistance of the pearl oyster to infection by external pathogenic microorganisms depends on innate immunity, and that the gill tissue plays an important role in immune response [33, 34]. Pathogenic bacteria can damage shellfish by the production of excessive reactive oxygen species and free radicals, which leads to tissue and cell damage, reducing the immune capability and lipid peroxidation of shellfish [35]. Therefore, improving the function of immune and antioxidant enzymes can effectively protect shellfish from pathogenic microorganisms.

Both AKP and ACP are important phosphatases whose functions are to remove excess proteins, carbohydrates, and lipids by removing phosphate groups from the body [36]. Furthermore, SOD and CAT are important in the initial antioxidant defense mechanism of shellfish [37, 38]. The T-AOC level reflects the general antioxidant capacity of the body and indicates its ability to cope with immune stress. Although LZM is a non-specific immune factor in the body, it can destroy and eliminate microorganisms and induce and regulate the synthesis and secretion of other immune factors [39]. *Pinctada fucata martensii* resists pathogen infection by improving its immune and antioxidant capacity. Therefore, this study analyzed the functions of the miRNAs *novel-13* and *novel-44* by comparing the activities of enzymes related

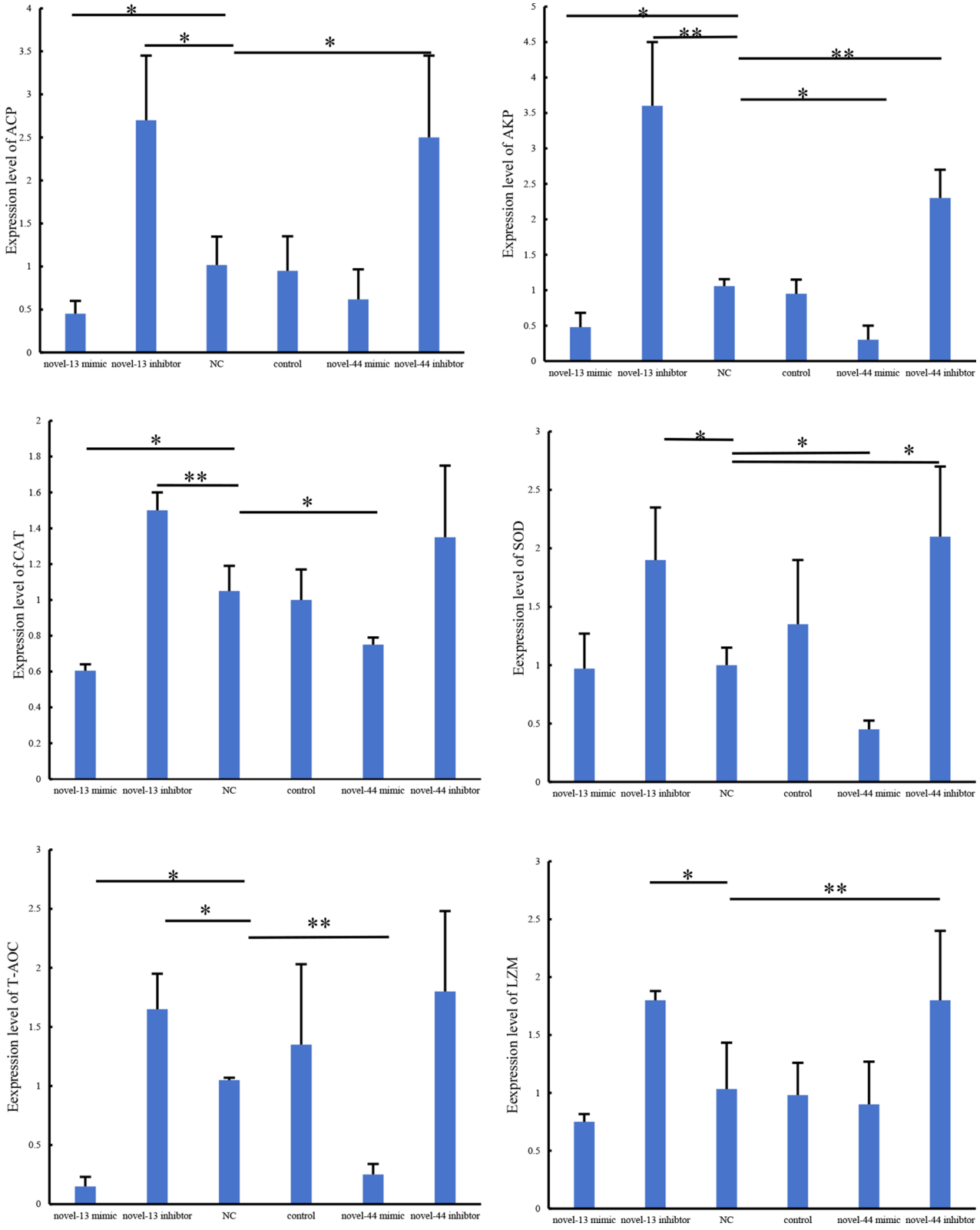

Fig. 5 Enzyme activity in *Pinctada fucata martensii* after overexpression and inhibition of miRNAs and stimulation by *Vibrio parahaemolyticus*. *Note*: Data are presented as mean ± SD ($N = 3$). * Represents a significant difference with the NC control ($p < 0.05$), ** represents a highly significant difference with the NC control ($p < 0.01$), Control: PBS control group

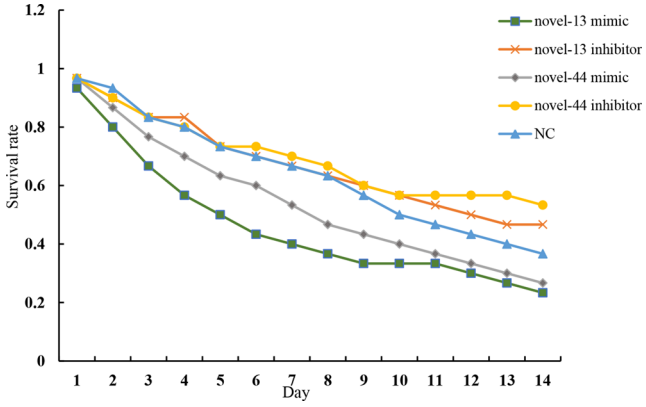

Fig. 6 *Pinctada fucata martensii* survival rates after different miRNAs injections and challenge with *Vibrio parahaemolyticus*. *Note*: Data are presented as mean ± SD (*N* = 3). Green represents the *novel-13* mimic group; red represents the *novel-13* inhibitor group; gray represents the *novel-44* mimic group; yellow represents the *novel-44* inhibitor group; and blue represents the NC group

to immunity and antioxidation between the healthy and *VP*-infected gill tissues of *P. f. martensii.* In healthy shellfish, the results showed that the *novel-13* and *novel-44* inhibitors significantly upregulate the enzyme activities in normal tissues, while the *novel-13* and *novel-44* mimics significantly downregulate the enzyme activities. As a kind of flavoprotease, *LAAO*, with flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as coenzyme, can specifically catalyze the oxidative deamination of L-amino acids to the corresponding α-keto acids, ammonia and H_2O_2, accompanied by oxygen consumption [40]. Due to the negative regulatory relationship between *novel-13* and *LAAO*, the expression of *LAAO* increased, leading to the accumulation of ammonia and peroxide in shellfish, thereby increasing the immune and antioxidant-related enzyme activities. Similarly, *ILK* regulates phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) [41], *NF-κB* [42], and other signaling pathways involved in cell growth and immunity. A large number of studies have shown that *NF-κB* is involved in the inflammation and immune response, which is also the most important role of the NF-κB signaling pathway in biology [43–45]. Li's study has shown that there is a negative regulatory relationship between the NF-κB signaling pathway and the Keap1-Nrf2 signaling pathway, possibly achieved through the direct interaction between P65 and Kelch-like ECH-associated protein 1 (Keap1) at the protein level [46]. Under stress, reactive oxygen species (ROS) modify the cysteine residues of Keap1, changing its conformation and allowing Nrf2 to be released into the nucleus [47]. Subsequently, the dimer formed by Nrf2 and the small Maf protein (sMaf) binds to antioxidant response elements (ARE) to activate the expression of downstream target genes and upregulate antioxidant enzymes [48]. Therefore, it is speculated that the increased activity of antioxidation-related enzymes induced by injection of the *novel-44* inhibitor may be due to the upregulation of its target gene *ILK*. Additionally, *ILK* may downregulate the expression of P65 in the NF-κB signaling pathway, thereby activating the Keap1-Nrf2 signaling pathway. Similarly, Dai [49] also showed that, compared with the NC control group, the injection of a *novel-63* mimic significantly reduced the activities of immune and antioxidation-related enzymes.

It is known that *VP* infection can cause the body to produce more ROS [50]. In a previous *VP*-stimulated transcriptome study, the expression rate of *novel-13* and *novel-44* was significantly downregulated [25]. To further explore the role of miRNAs in *VP* infection in this study, *VP* was injected after the injection of inhibitors and mimics of the two miRNAs. Consistent with the healthy shellfish injection experiment, the inhibitor group significantly upregulated the activities of immune and antioxidant-related enzymes. Also, the results of the survival experiment showed that the survival rate of the group injected with the miRNA inhibitors was higher than that of the control group and mimics injection group. The above results suggest that the increased expression of the two miRNAs can reduce the activities of immune and antioxidant-related enzymes and that this regulation may be achieved through the regulation of their target genes. After the injection of the miRNA inhibitors, the reinfection of *VP* could upregulate the activities of the immune and antioxidant enzymes and improve the survival rate of *P. f. martensii*.

Conclusions

Due to the economic value of cultivated pearl oysters and their continuous susceptible health in these cultured environments, research into their immune function is justified. This work showed that there was a negative regulatory relationship between the *novel-13* and *novel-44* and their target genes. Injection of both miRNA mimics and inhibitors before and after *VP* infection caused

changes in the activities of immune and antioxidant-related enzymes in *P. f. martensii*. Injection of inhibitors could improve the survival of *P. f. martensii* infected with *VP*. These results indicate that *novel-13* and *novel-44* play an important role in the response to *VP* infection, and provide a theoretical basis for further study of the miRNA-mRNA immune regulatory network of *P. f. martensii*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04467-0.

Supplementary Material 1

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Author contributions
Li completed the first draft of the paper. Z completed the experiment. Liang provided funding and completed the final draft of the paper. Z and M completed the data processing. S provided the experimental material.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The experimental animals involved in this study have been approved and agreed by the Animal Experiment Ethics Committee of Guangdong Ocean University.

Consent for publication
Not applicable.

Human ethics and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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